Interaction between Ferredoxin and Ferredoxin Nicotinamide Adenine Dinucleotide Phosphate Reductase in Pyridine Nucleotide Photoreduction and Some Partial Reactions

I. INHIBITION OF FERREDOXIN NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE REDUCTASE BY FERREDOXIN

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SUMMARY

Purified ferredoxin has been shown to inhibit reactions mediated by the flavoprotein ferredoxin-NADP reductase. Ferredoxin inhibits the transfer of electrons from NADPH to ferricyanide (diaphorase activity) to NAD (transhydrogenase) and the photoreduction of pyridine nucleotides during the Hill reaction. On the basis of the kinetics of inhibition, it is suggested that the flavoprotein has two binding sites for substrates. At one site the enzyme binds NADPH and at the other site NADP or NAD. Ferredoxin inhibits the flavoprotein by competing with the binding of substrates at both sites.

At proper ferredoxin concentrations, NAD can be photoreduced by isolated chloroplasts at a rate of 50 umoles per mg of chlorophyll per hour. Since chloroplasts isolated by conventional methods have lost part of the ferredoxin-NADP reductase it is suggested that NAD might be photoreduced at appreciable rates in vivo.

The photochemical reduction of pyridine nucleotides in isolated chloroplasts has been shown to proceed via ferredoxin and ferredoxin-NADP reductase (EC 1.6.99.4) (1). The latter enzyme is a flavoprotein which can also catalyze the transfer of electrons “in reverse,” from NADPH to ferricyanide (2) or to other acceptors such as ferricyanide and trichloroindophenol (3), NAD (4), pyocyanine (5), low potential viologen dyes (5, 6), plastocyanine (7), and cytochrome f but not cytochrome c (8). The reduction of chloroplast cytochrome f was suggested to be involved in cyclic electron flow (8).

In the present study it has been found that purified ferredoxin inhibits all the reactions catalyzed by the flavoprotein, including the photoreduction of pyridine nucleotides. The photoreduction of NAD was found to be highly susceptible to this inhibition, but in the presence of appropriate ferredoxin concentrations NAD was reduced at appreciable rates. Thus the currently accepted view that only NADP is reduced in photosynthesis (9) should be questioned.

A preliminary report of part of this work has been published (10).

MATERIALS AND METHODS

Preparation of Enzymes—The flavoprotein ferredoxin-NADP reductase was prepared from Swiss chard or lettuce chloroplasts and purified according to the procedure of Shin, Tagawa, and Arnon (1). The purified protein had a ratio of absorbance at 456 nm to 275 nm of 0.085. Ferredoxin was isolated from Swiss chard leaves and purified essentially according to the procedure of Boger, Black, and San Pietro (11), omitting the protamine sulphate step which was replaced by an additional adsorption on a DEAE-cellulose column and elution with 0.35 M NaCl-0.05 M Tris, pH 8.0. The purified ferredoxin had a ratio of absorbance at 330 nm to that at 277 nm of 0.60. The concentrations of the purified proteins were calculated on the basis of the following molar extinction coefficients: 8,240 at 465 nm for ferredoxin (12) and 10,740 at 456 nm for the flavoprotein (13).

Assays—The diaphorase activity of both the purified flavoprotein and the chloroplast-bound enzyme was measured either by ferricyanide reduction or by oxygen uptake in the presence of methyl viologen with a Gilson Oxygraph (Gilson Medical Electronics, Middleton, Wisconsin) equipped with a Yellow Spring Instruments Clark type of oxygen electrode. The transhydrogenase reaction was measured by the increase in absorbance at 340 nm which was the result of NAD reduction, since the NADPH concentration was kept constant with a regenerating system of glucose 6-phosphate, NADP, and glucose 6-phosphate dehydrogenase. Cytochrome c reduction was measured by the increase in absorption at 550 nm,

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The effect of heating on ferredoxin activity in NADP photoreduction and in transhydrogenase inhibition. Purified ferredoxin at $2 \times 10^{-4}$ M in 5 mM Tris, pH 8.0, was incubated at 50°C for various time intervals. The reaction mixture for NADP photoreduction included the following in micromoles: Tris, 20, at pH 8.0; NaCl, 40; NADP, 0.25; and ferredoxin, 0.004. Chloroplasts equivalent to 5 mg of chlorophyll were added in a total volume of 1.0 ml. NADP photoreduction was measured by Procedure a (see “Assays”). The reaction mixture for transhydrogenase activity included the following in micromoles: Tris, 50, at pH 8.0; glucose 6-phosphate, 5; NADP, 0.001; NAD, 2.5; and ferredoxin, 0.004. Glucose 6-phosphate dehydrogenase (1 unit) and the purified flavoprotein (10 pg) were also present. The incubation was carried on for 4 min. The control value for transhydrogenase activity with no ferredoxin was 11.6 umoles of NAD reduced per min. O, NADP photoreduction; •, transhydrogenase activity.

assuming a millimolar extinction coefficient (reduced minus oxidized) of 18.5.

The photoreduction experiments were performed with lettuce chloroplasts except when stated otherwise. The chloroplasts were isolated as described before (14) except that serum albumin at a concentration of 1 mg per ml (which was shown to improve photoreactions (15)) was present during homogenization. The chloroplasts were “fragmented” by suspension in 10^{-3} M Tris, pH 8.0. After centrifugation they were resuspended in the original medium, to which serum albumin was added to a final concentration of 10 mg per ml. Pyridine nucleotide photoreduction was performed by two slightly different procedures. (a) The reaction mixture was illuminated for 3 min by a 150-watt bulb, shielded through a water bath, which provided 3500 foot-candles at the level of the test tubes. To stop the reaction, the light was turned off and the increase in absorbancy at 340 nm was measured with an Uvispec spectrophotometer, assuming a millimolar extinction coefficient of 6.2. (b) The absorbance at 350 nm was recorded continuously (with a millimolar extinction coefficient of 5.85 for NADPH) in a Cary 15 spectrophotometer. In the latter procedure the actinic beam was provided by a 300-watt slide projector at right angles to the measuring beam and passed through a red filter (Corning No. 2403). The phototube was protected from the actinic beam by a blue filter (Corning No. 5874) and a saturated solution of CuSO4. The light intensity before passing through the red filter was approximately 10,000 foot-candles.

Materials—Glucose 6-phosphate dehydrogenase, glucose 6-phosphate, and pyridine nucleotides were obtained either from Boehringer or from Sigma. Cytochrome c (prepared from horse heart) was obtained from Boehringer. All other chemicals were of analytical grade.

RESULTS

Inhibition by Ferredoxin of Partial Reactions Catalyzed by Chloroplast Flavoprotein—In a previous communication (10) we have shown that ferredoxin inhibited the diaphorase activity of the flavoprotein. In Fig. 1, it is shown that ferredoxin heated for 10 min at 50°C does not inhibit, and the decrease of the inhibitory activity of ferredoxin in the transhydrogenase reaction was paralleled by the loss of ferredoxin activity in photoreduction.

When ferredoxin was acidified to pH 3.1 with HCl, again both activities were abolished (data not shown). In this experiment chloroplast-bound flavoprotein was used as a control; thus it is clear that ferredoxin inhibits the flavoprotein whether purified or grana-bound.

As shown previously (10), ferredoxin inhibits the diaphorase competitively with NADPH. That experiment was performed with one concentration of ferredoxin (7.2 µM) only, because higher ferredoxin concentrations chemically reduce ferricyanide. As shown in Fig 2 (with two concentrations of ferredoxin), ferre...
FIG. 3. The effect of ferredoxin on the transhydrogenase activity at various NADP and NAD concentrations. The reaction mixture of Experiments A and B contained the following in micromoles: Tris, 75, at pH 8.0; glucose 6-phosphate, 5; and ferredoxin, where indicated, 0.0033; 1 unit of glucose 6-phosphate dehydrogenase and 12 μg of flavoprotein in a total volume of 1.5 ml. In A 0.001 μmole of NADP was added, and NAD as specified. In B 1.5 μmole of NAD was added and NADP as specified. The reaction mixture of Experiments C and D contained the following in micromoles: Tris, pH 8.0, 50; glucose 6-phosphate, 5; and ferredoxin, 0.0048, when indicated; glucose 6-phosphate dehydrogenase (1 unit) and flavoprotein (15 μg) in a total volume of 1.0 ml. In C 0.05 μmole of NADP was added and different amounts of NAD as specified. In D 5 μmole of NAD was added and various amounts of NADP. The velocity is expressed in millimicromoles of NAD reduced per min. ●, plus ferredoxin; ○, control.

Ferredoxin also inhibited the diaphorase competitively with NADPH when methyl viologen served as the electron acceptor. The calculated $K_m$ for NADPH in this reaction was 6 μM, and the $K_i$ for ferredoxin was 3.5 and 3.0 μM in the presence of 7 and 14 μM ferredoxin, respectively.

Ferredoxin was found to inhibit also the transhydrogenase reaction, i.e. the transfer of electrons from NADPH to NAD (Fig. 1). Low concentrations of ferredoxin somewhat stimulated this activity, but the stimulation has not been studied further. The results presented in Fig. 3 indicate that, in the transhydrogenase reaction, the type of inhibition obtained depended on substrate concentration. At a low NADPH concentration (0.07 μM) and varying concentrations of NAD, the inhibition obtained was of a "mixed" type (16), i.e. $V_{max}$ is decreased and $K_m$ increased (Fig. 3A). A similar pattern of inhibition, decrease in $V_{max}$ and increase in $K_m$, is obtained in the presence of a relatively low NAD concentration (1 mM) and varying NADP concentrations (Fig. 3B). However, in the presence of a saturating concentration of one of the substrates, the inhibition by ferredoxin with respect to the concentration of the second substrate was strictly competitive (Fig. 3, C and D).

Lazzarini and San Pietro (2) have shown that the flavoprotein can catalyze the transfer of electrons from NADPH to ferredoxin, which chemically reduces cytochrome c. As can be seen in Fig. 4, this reaction was inhibited by NADP competitively with ferredoxin.

### Photoreduction of Pyridine Nucleotides

The data of Fig. 5 show that NADPH inhibited the photoreduction of NADP competitively with ferredoxin. This is expected on the assumption that ferredoxin and NADPH compete for a site on the flavoprotein (Figs. 2 and 3D). Increasing the concentration of NADP, on the other hand, did not cause inhibition of NADP photoreduction, nor did it eliminate the inhibition by NADPH.

Since ferredoxin was shown to inhibit the transhydrogenase also at the site where the acceptor (NAD) binds, it was of interest to find out whether the photoreduction of pyridine nucleotides by chloroplasts might also be inhibited by ferredoxin. We have shown in a previous report (10) that the photoreduction of both NADP and NAD is inhibited by high concentrations of ferredoxin. NAD photoreduction is more susceptible to this inhibition than NADP (Fig. 6 and Reference 10). In the experiment described in Fig. 6, pea chloroplasts have been used because they contain less flavoprotein (Table I; see also Reference (5)) and therefore reduce NAD only upon addition of exogenous flavoprotein. In this case too, however, high concentrations of ferredoxin inhibited.

The inhibition of pyridine nucleotide photoreduction by high concentrations of ferredoxin was seen also when ascorbate-2,6-dichloroindophenol (in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea) was used as the electron donor (18) instead of water.

It is noteworthy that not only the rate of NAD photoreduction but also the $K_m$ for NAD in this reaction depends on ferredoxin concentration. When the latter was lowered 10-fold (from 4.5 μM to 0.45 μM), the $K_m$ for NAD decreased from 6.5 mM to 0.8 mM (Fig. 7).

The release of the flavoprotein from chloroplasts during isola-
FIG. 5. The effect of NADPH on NADP photoreduction at various ferredoxin concentrations. The reaction mixture contained in micromoles the following: Tris, pH 8.0, 20; NaCl, 35 NADP, 0.25; and NADPH, when present, 0.08. It also contained chloroplasts equivalent to 15 μg of chlorophyll and ferredoxin as indicated, in a total volume of 1.0 ml. NADP reduction was followed by Procedure a. ●, NADPH added; ○, control.

FIG. 6. Photoreduction of pyridine nucleotides by pea chloroplasts. The reaction mixture contained in micromoles the following: Tris, pH 8.0, 50; NaCl, 70; NAD, NADP, 0.25, where indicated; and NAD, NADP, 0.9, where indicated. It also contained chloroplasts equivalent to 15 μg of chlorophyll and ferredoxin as indicated, in a total volume of 1.0 ml. NADP reduction was followed by Procedure b. ◻, ○, no flavoprotein added; ■, ●, plus flavoprotein (10 μg). ◻, ■, NAD photoreduction; ○, ●, NADP photoreduction.

FIG. 7. The $K_m$ of NAD in photoreduction. The reaction mixture contained in a total volume of 1.5 ml the following in micromoles: Tris, pH 8.0, 50; NaCl, 70; NAD, as indicated, and chloroplasts equivalent to 14.5 μg of chlorophyll. ○, 0.00087 μmole of ferredoxin added; ●, 0.00087 μmole of ferredoxin added. NAD photoreduction was followed by recording continuously the absorbance at 350 nm in a Cary 15 spectrophotometer.

**Table I**

**Release of chloroplast flavoprotein**

Pea leaves (7 g) or lettuce leaves (23 g) were homogenized in 80 ml of solution as described (14) but in the presence of 1 mM ascorbate. The homogenate was centrifuged for 10 min at 2,500 X g. The first chloroplast pellet was resuspended in 80 ml of 2 mM Tris, pH 8.0, and again centrifuged for 10 min at 20,000 X g to obtain the second chloroplast pellet. Diaphorase activity was assayed as in Reference 10 except that NADP was added at 0.1 mM. Since various diaphorases are present in the leaf homogenate, only that enzyme which was inhabitated by an antibody specific for the chloroplast flavoprotein was taken into account. The antibody was prepared against a purified chloroplast diaphorase according to Reuter, San Pietro, and Stolzenbach (17).

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**DISCUSSION**

Purified ferredoxin was shown to inhibit reactions mediated by ferredoxin-NADP reductase. The inhibition depends on the intactness and proper conformation of ferredoxin, since heating...
reduction of ferredoxin (as visualized by cytochrome c reduction) or NADPH (in the transhydrogenase reaction and in the Hill reaction (Fig. 5). In the latter, NADPH inhibits the rate of reduction of NADP competitively with ferredoxin. On the other hand, increasing the concentration of ferredoxin 5-fold has no effect on either the NADP photoreduction or the inhibition by NADPH. At Site B, ferredoxin (presumably reduced) and NADPH is clearly seen in the diaphorase reaction (Fig. 2), the transhydrogenase reaction (Fig. 3D), and in the Hill reaction (Fig. 5). In the latter, NADPH inhibits the rate of reduction of NADP competitively with ferredoxin. On the other hand, increasing the concentration of NADP 5-fold has no effect on either the NADP photoreduction or the inhibition by NADPH. At Site B, ferredoxin (presumably oxidized) inhibits the reduction of pyridine nucleotides during the Hill reaction (Fig. 6 and Reference 10) and the transhydrogenase, competitively with NAD (Fig. 3C). At this site, the reduction of ferredoxin (as visualized by cytochrome c reduction) is inhibited by NADP (Fig. 4). The high affinity of the flavoprotein for NADP during NADP reduction (9) and for NADPH in the transhydrogenase (4) is compatible with our proposed model that those two substrates bind at different sites on the enzyme. Also in agreement with this model is the fact that the transhydrogenase shows normal saturation kinetics as a function of either substrate. If, on the other hand, NADPH and NAD would have been bound at the same site, an optimum curve should have been obtained. Dyes probably accept electrons from a different site, perhaps directly from reduced FAD (see also Reference 8).

The importance of NAD compared with NADP as the final hydrogen acceptor in the Hill reaction has been studied extensively. In early studies with no enzymes added, a high grana-concentration and long incubation times, NAD was reduced much faster than NADP (20). Upon addition of a crude leaf fraction which contained ferredoxin and flavoprotein, NADP was reduced somewhat faster than NAD (21). Subsequently, it was shown (4) that the reduction of both pyridine nucleotides depends on the presence of ferredoxin, but NAD reduction requires flavoprotein as well. This led to the suggestion that NADP is reduced by ferredoxin directly, whereas NAD accepts hydrogens from NADPH by flavoprotein acting as transhydrogenase (4). However, it was suggested by Lazzarini and San Pietro (2) and shown by Shin and Arnon (9) that the chloroplast flavoprotein participates in the reduction of both pyridine nucleotides. By comparing the affinities of the two nucleotides and the rate of reduction, the latter authors concluded that NAD photoreduction has no physiological significance. The stimulation of NAD reduction by externally added flavoprotein found previously by Keister, San Pietro, and Stolzenbach (4) was not discussed.

Our data support essentially those of Shin and Arnon (9), showing that both pyridine nucleotides are reduced directly by the flavoprotein at the same site and that NADP is reduced at a faster rate than NAD. However, we have shown that by adding proper ferredoxin concentrations, respectable rates of NAD reduction can be obtained and the $K_m$ for NAD is lowered. It is interesting that, whereas the ratio of ferredoxin to chlorophyll in the chloroplasts is probably 1:400 (22), that required in the reaction mixture for optimal NADP photoreduction with isolated chloroplasts in approximately 1:1 (10). Moreover, isolated lettuce chloroplasts in which the bound flavoprotein does not limit NADP photoreduction have lost about 50% of the original enzyme. This loss is more striking in isolated pea chloroplasts because the flavoprotein is bound less tenaciously than in pea chloroplasts. The stimulation of NAD reduction by externally added flavoprotein does not limit NADP photoreduction unless it is reduced at a faster rate than NAD. Moreover, we have shown that by adding proper ferredoxin concentrations, respectable rates of NAD reduction can be obtained and the $K_m$ for NAD is lowered. It is interesting that, whereas the ratio of ferredoxin to chlorophyll in the chloroplasts is probably 1:400 (22), that required in the reaction mixture for optimal NADP photoreduction with isolated chloroplasts in approximately 1:1 (10). Moreover, isolated lettuce chloroplasts in which the bound flavoprotein does not limit NADP photoreduction have lost about 50% of the original enzyme. This loss is more striking in isolated pea chloroplasts because the flavoprotein is bound less tenaciously to the chloroplast membranes (Fig. 6 and Table 1). Consequently, in vivo, in the presence of the full amount of chloroplast flavoprotein and no excess of ferredoxin, NAD might be reduced at appreciable rates. Studies of pyridine nucleotides reduction in leaves also support this notion (23).

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REFERENCES
Interaction between Ferredoxin and Ferredoxin Nicotinamide Adenine Dinucleotide Phosphate Reductase in Pyridine Nucleotide Photoreduction and Some Partial Reactions: I. INHIBITION OF FERREDOXIN NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE REDUCTASE BY FERREDOXIN

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